nitrogenase. Nitrogen fixation by *C. cellobioparum* and *C. thermocellum* during growth on cellulose was not reported.

Inasmuch as environments rich in cellulose are frequently deficient in nitrogen (for example, peat soils, agricultural and municipal wastes, and composts), cellulose-fermenting bacteria that satisfy their nitrogen requirements through the fixation of N_2 would be expected to have a strong selective advantage over those that require a source of combined nitrogen. Because vast amounts of cellulose are available in a wide variety of environments, it is possible that cellulolytic, nitrogen-fixing bacteria are widespread in nature and that they play a significant role in nitrogen and carbon cycling. The potential use of these bacteria in improving soil fertility and in the conversion of lignocellulosic wastes into useful products remains to be explored.

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An in Vitro System for Accurate Methylation of Internal Adenosine Residues in Messenger RNA

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Some internal adenosine residues in messenger RNA are methylated posttranscriptionally in the nucleus. Most of the methylated adenosine residues in prolactin mRNA are in the 3′ untranslated region. The site of methylation in the 3′ end of prolactin mRNA was determined. This methylation reaction is highly specific; of the three adenosine residues in consensus sequences located in the 3′ end, only one is methylated. An in vitro methylation system was developed in which bovine prolactin mRNA, synthesized in vitro with T7 RNA polymerase, was accurately methylated in a HeLa cell nuclear extract. The adenosine residue that was methylated in vitro was the same as the one methylated in vivo. This cell-free system, which accurately methylates the N^6-position of adenosine residues in mRNA, will allow further study of the mechanism of adenosine methylation.

N^6-METHYLADENOSINE (m^6A) IS A posttranscriptional modification of adenosine that occurs in most eukaryotic cellular and viral mRNAs with one to three m^6A residues per 1000 nucleotides (*1–3*). The methylated adenosine is usually found within an AAC or GAC consensus sequence (*4–6*). Localization studies performed on a few specific mRNAs indicate that the distribution of m^6A is nonrandom. For example, in Rous sarcoma virus genomic RNA there are 10 to 15 m^6A residues clustered in the 3′ end of the RNA (*3*). Similarly, in bovine prolactin mRNA, most of the m^6A residues are located in the 3′ untranslated sequence of 138 nucleotides (*7*). This region of prolactin mRNA contains only 3 consensus sequences of a total of 27 sequences present throughout the mRNA. Therefore, the site of methylation appears to be specific and perhaps dictated by more than just the 3-nucleotide consensus sequence. However, the mechanism and function underlying this modification of mRNA remains obscure.

In an attempt to study the mechanism of m^6A formation in mRNA molecules we established an in vitro methylation system with synthetic mRNA substrates and HeLa cell nuclear extracts. Bovine prolactin mRNA was synthesized in vitro with T7 RNA polymerase (Fig. 1A) and incubated with a HeLa cell nuclear extract in the presence of the methyl donor S-[^{3}H]adenosylmethionine (SAM). Under the conditions described in the legend to Fig. 1B, 70 to 80% of the radioactive methyl groups were incorporated into internal adenosine residues (Fig. 1B) and 5% of the input RNA was methylated. High-performance liquid chromatography (HPLC) showed that the predominant methylated component is m^6A with a low level (7% to 8%) of the methylated nucleotides as 2′-O-methyl-

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uridine (U_m). Methylation was observed as early as 5 min after incubation and increased linearly up to 30 min (*8*). Analysis of 314 nucleotides at the 5′ end and 138 nucleotides at the 3′ end of the RNA (Fig. 1A) indicated that there was preferential methylation in the 3′ fragment and the low level of U_m generated in vitro was present only in this sequence (*8*). Therefore, the HeLa cell nuclear extract can methylate prolactin mRNA with the same specificity as that observed in vivo.

The precise location of m^6A residues in the 138-nucleotide fragment of prolactin mRNA methylated in vitro was determined by a one-dimensional analysis of a discrete set of 15 oligonucleotides obtained by T1 digestion of the 3′ end fragment. Because the three consensus sequences of $\overset{A}{G}AC$ present in this fragment are located in three unique oligonucleotides of different sizes (Fig. 2A), it is possible to determine if one or more of the consensus sequences are methylated.

Analysis of unmethylated (^{14}C-labeled) RNA showed that the largest oligonucleotide is 21 nucleotides long and the smallest is 3 nucleotides long (lane 2 in Fig. 2B). Smaller oligonucleotides of different base composition but same size (for example, the 5-base fragments, 5′ and 5″) are separated. The difference in the intensity of T1 oligonucleotides reflects the difference in their uridine content. Analysis of the methylated RNA (^3H-labeled) indicated that only 2 of the 15 oligonucleotides are methylated; the 6-base fragment and one of the two 5-base fragments (lane 1 in Fig. 2B). The 21-base and 12-base fragments, both of which contain the consensus AAC sequence, are not methylated in this in vitro reaction.

To test the hypothesis that the slight difference in migration between the methylated and unmethylated 6-base fragment is due to the addition of a methyl group, we synthesized prolactin mRNA in the presence of [^{14}C]uridine triphosphate (UTP). The T1 oligonucleotides from the 3′ end fragment were compared before and after methylation with [^3H]SAM. In methylated RNA, two 6-base oligonucleotide bands were observed with the same difference in mobility observed in Fig. 2B. The unmethylated RNA contained only one 6-base oligonucleotide (*8*).

To verify the identity of the 6-base fragment by a property other than size as predicted by gel mobility, the full-length pro-

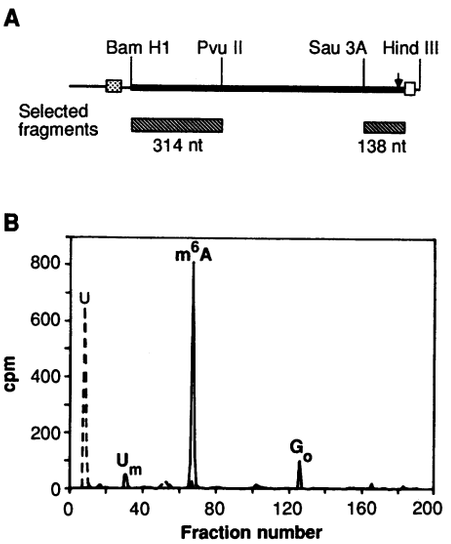

Fig. 1. N^6-methylation of adenosine residues in vitro. (**A**) Structure of the DNA template used for in vitro transcription of full-length prolactin mRNA. The stippled box indicates the T7 promoter, the thin line indicates the vector sequences, and the thick line indicates the bovine prolactin cDNA (894 nucleotides). The polyadenylation site is indicated by the arrow and the white box indicates the cytosine residues (28 nucleotides) from GC tailing of the original cDNA library. The guanosine residues at the 5′ end of the original cDNA were deleted by oligonucleotide-directed mutagenesis (*13*). The plasmid was linearized with Hind III. RNA containing a 5′ terminal cap was transcribed from the T7 promoter in a reaction containing a fivefold excess of G1 cap (m^7GpppG_m) and low levels of [α-^{32}P]UTP or [^{14}C]UTP, or in the absence of radioisotopes. The 314-nucleotide fragment at the 5′ end and 138-nucleotide fragment at the 3′ end (hatched boxes) were selected by filter hybridization. (**B**) Methylation of full-length prolactin mRNA and HPLC analysis. Capped RNA (2 to 3 μg) was methylated in vitro in a 50-μl reaction containing 24 m*M* Hepes *p*H 7.9, 100 m*M* KCl, 4 m*M* $MgCl_2$, 1.1 m*M* dithiothreitol (DTT), 4% glycerol, 0.04 m*M* EDTA, 3% polyvinyl alcohol, 10 μl of HeLa cell nuclear extract (*14*), and 2 μ*M* SAM (8 μCi, 80 Ci/mmol). After incubation at 30°C for 30 min, the reaction was diluted with an equal volume of 100 m*M* tris-HCl, *p*H 7.6, 20 m*M* EDTA, 20 m*M* NaCl, and 0.4% SDS and treated with 50 μg of proteinase K. The reaction was subsequently extracted with phenol:chloroform (1:1) and precipitated with ethanol. Methylated prolactin RNA was selected by filter hybridization (*15*) and analyzed by HPLC with the use of a system that resolves all the methylated constituents (cap structures and nucleosides) found in mRNA (*16*). The broken line shows the peak of [^{14}C]uridine. G_o, m^7GpppG.

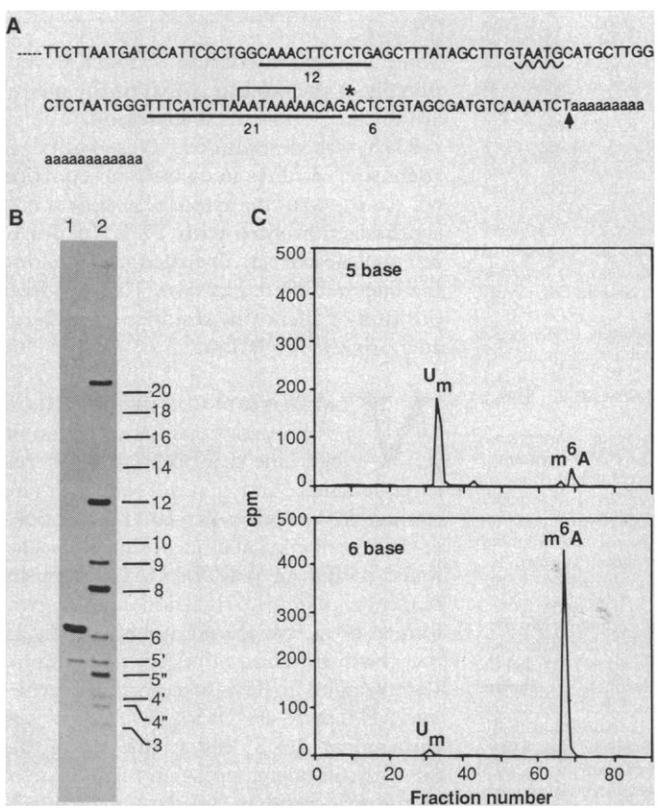

Fig. 2. T1 oligonucleotide analysis of the 3′ end of prolactin transcript methylated in vitro. Full-length prolactin RNA was methylated in vitro, and 138 nucleotides at the 3′ end were selected by hybridization to M13 DNAs containing prolactin 3′ sequences (*15*). After hybridization, filters were treated with T1 RNase (1 μg/μl) at 37°C for 30 min to digest unhybridized RNA. Filters were washed (*17*), and the RNA was eluted (*15*). (**A**) Sequence of 138 nucleotides from the 3′ end of bovine prolactin mRNA. The oligonucleotides containing the consensus sequences are underlined, and their respective lengths are indicated. The methylated adenosine is indicated by an asterisk. The methylated 5-base fragment containing U_m is indicated by the wavy underline. The consensus hexanucleotide and site of polyadenylation are indicated by the bracket and arrow, respectively. The 21 adenosine residues that follow the polyadenylation site are also shown. (**B**) The 3′ terminus (138-nucleotide) of unmethylated (^{14}C-labeled) and methylated (^3H-labeled) prolactin mRNA were digested separately with T1 RNase (2 μg) in the presence of 10 μg of yeast transfer RNA at 37°C for 45 min. T1 oligonucleotides were separated by electrophoresis on a 20% polyacrylamide/7*M* urea gel. The oligonucleotides were transferred to Zetaprobe membrane (Bio-Rad), sprayed with EN^{3}HANCE (Du Pont, Biotechnology Systems), and analyzed by fluorography. Lane 1, methylated RNA; lane 2, unmethylated RNA. The lengths of the oligonucleotides were determined by comparison with an RNA ladder (generated by alkaline hydrolysis of 3′ end-labeled RNA) analyzed in parallel (*8*). (**C**) Determination of methylated nucleosides in 5- and 6-base fragments. The two oligonucleotides (5 and 6) were eluted separately from the gel (*12*), digested to nucleosides (*15*), and analyzed by HPLC.

lactin transcript was synthesized with [α-^{32}P]adenosine triphosphate (ATP). Because the 6-base fragment has only one adenosine residue and this adenosine is found as the first residue in the oligonucleotide (Fig. 2A), T1 digestion of the [α-^{32}P]ATP–labeled prolactin 138-nucleotide fragment should result in the loss of label from the 6-base fragment [T1 ribonuclease (RNase) generates 3′ phosphorylated oligonucleotides]. The results of this analysis confirmed that the methylated oligonucleotide was the 6-base fragment with the sequence ACUCUG (*8*).

In contrast to the 6-base oligonucleotide, the 5-base fragment lacks a consensus sequence and therefore probably does not contain m^{6}A. This was verified by elution of the 5-base and 6-base fragments from the gel, digestion to nucleosides, and HPLC analysis. The methylated nucleoside in the 6-base fragment is m^{6}A, whereas U_m is the modified nucleoside in the 5-base fragment (Fig. 2C). We do not know if one or both of the uridine residues present in this oligonucleotide are modified. This finding was unexpected because U_m has not been previously observed in internal positions in mRNA. The significance of U_m in prolactin mRNA methylated in vitro and of its possible presence in vivo remains to be determined.

The exact site of m^{6}A methylation in vivo in bovine prolactin mRNA had not been previously determined. This was accomplished by T1 oligonucleotide analysis of prolactin mRNA derived from the bovine pituitary. Identical T1 oligonucleotide patterns obtained from the 3′ end of pituitary prolactin mRNA (lane 2 in Fig. 3A) and in vitro–synthesized prolactin RNA (lane 1 in Fig. 3A) indicated that the mRNA selected from the pituitary is the 3′ end of prolactin mRNA and is essentially free of other mRNAs. Two-dimensional thin-layer chromatography (TLC) analysis was performed on 5′ phosphorylated mononucleotides derived from six different T1 oligonucleotides found in the 3′ terminal region of prolactin mRNA, two of which are shown in Fig. 3B. Comparison of the nucleotides in the two oligonucleotides shows that m^{6}A is only present in the 6-base fragment and not in the 12-base fragment. Analysis of the 21-, 8-, 5-, and 4-base fragments also indicated a complete absence of m^{6}A (*8*). Only one of the three adenosine residues present within consensus sequences in the 3′ end of the mRNA is methylated, thereby indicating that the methylation reaction is highly specific. In addition, the same adenosine residue is methylated in vivo and in vitro. Because there is a single adenosine residue in the 6-base fragment (Fig. 2A), it is possible to determine the fraction of methylated adenosine residues at this site in vivo. We accomplished this by eluting areas of the TLC plate corresponding to A and m^{6}A residues and by determining their relative radioactivity. Approximately 20% of adenosine residues are methylated; thus only one in every five mRNA molecules is methylated at this site. Two additional unidentified spots, which arise from the kinase reaction, are routinely seen in the TLC analysis of all the oligonucleotides.

In summary, we have established an in vitro methylation system that modifies mRNA posttranscriptionally by adding methyl groups to the N^6 position of internal adenosine residues and maintains the specificity of the in vivo reaction. We have also determined the precise adenosine residue that is methylated in the 3′ terminus of prolactin mRNA in vivo. The specificity of m^{6}A methylation in the 3′ terminal segment of prolactin mRNA both in vivo and in vitro is remarkable. It has been shown that in Rous sarcoma virus genomic RNA, PuGACU sequences were methylated more frequently than AAC sequences (*9*). The methylated sequence in prolactin mRNA is AGACU, which suggests that this longer pentanucleotide may be the preferred consensus sequence. As observed in the methylation of Rous sarcoma virus RNA (*9*), methylation of the AGACU sequence in prolactin mRNA was also nonstoichiometric. One explanation for this heterogeneity is that the methyl groups are removed from steady-state cytoplasmic mRNA by a demethylase activity present in the cytoplasm (*10*). Alternatively, several conformations of the RNA sequence may exist, only one of which provides an adequate configuration for modification by the methyltransferase. A possible corollary is that the potential functional role of N^6-methylation is expressed or required in only one of these possible conformations.

It is also interesting that the prolactin transcript used in the in vitro reaction is the mature mRNA and not the intron-containing precursor RNA. Even though methylation is believed to occur before splicing in vivo (*11*), the methyltransferase is capable of recognizing and accurately methylating mature mRNA. However, it may do so less efficiently than with its true substrate in vivo, which is the precursor RNA. This may explain the lower efficiency of methylation observed in vitro (5%) compared to methylation in pituitary prolactin mRNA (20%).

Studies on N^6-methylation of mRNA have been delayed because no in vitro system has been available. The ability to examine this modification in vitro with the use of nuclear extracts now makes it possible to study the mechanism of this processing event in detail. This system will enable us to study (i) the substrate requirements of this reaction, (ii) the possible requirement of an extended consensus sequence, and (iii) the role of the secondary structure in the RNA substrate. In addition, because this extract has the ability to splice and polyadenylate exogenous substrates (*12*), it will allow us to determine the effect of methylation on these processes. It is interesting that the methylated adenosine is just downstream of the polyadenylation consensus sequence and therefore it is tempting to speculate that

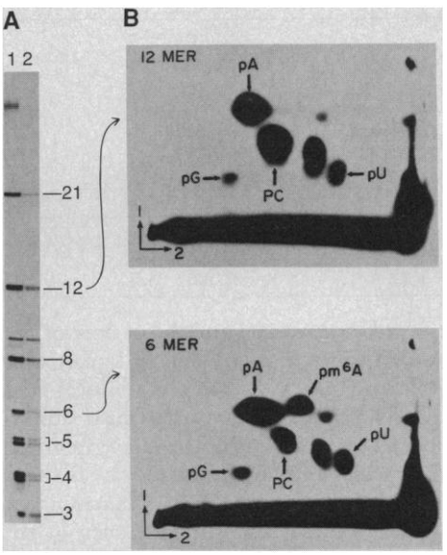

Fig. 3. Analysis of T1 oligonucleotides from pituitary prolactin mRNA. (**A**) To confirm that the pituitary-derived oligonucleotides are of prolactin origin the 3′ end (25 to 30 ng) of hybrid-selected prolactin mRNA or in vitro–synthesized prolactin RNA (unlabeled) was digested with 20 ng of T1 RNase, and the 5′ termini of the resulting oligonucleotides were labeled with [α-^{32}P]ATP and polynucleotide kinase. The oligonucleotides were separated by electrophoresis on a 20% polyacrylamide/7*M* urea gel. T1 oligonucleotides from in vitro–synthesized prolactin transcript (lane 1) and from pituitary prolactin mRNA (lane 2) are shown. (**B**) To identify the oligonucleotide, or oligonucleotides, that contain m^{6}A, the 3′ end fragment (100 to 200 ng) of pituitary prolactin mRNA was digested with 100 ng of T1 RNase, and the T1 oligonucleotides were separated by gel electrophoresis. The position of the unlabeled oligonucleotides on the gel was determined by comparison with the position of ^{32}P-labeled oligonucleotides analyzed in parallel. The oligonucleotides were eluted from the gel, hydrolyzed to mononucleotides with 0.1*N* NaOH at 65°C for 30 min, and labeled at the 5′ position with [α-^{32}P]ATP (*7*). After removal of the 3′ phosphate with P1 nuclease, the 5′ monophosphates were analyzed by two-dimensional TLC (*7*). The streak across the bottom represents free [α-^{32}P]ATP. TLC analysis of the 12-base (12 mer) fragment (top) and 6-base fragment (6 mer) (bottom) are shown. Abbreviations: pA, adenosine 5′-monophosphate; pG, guanosine 5′-monophosphate; pU, uridine 5′-monophosphate; PC, cytidine 5′-monophosphate; pm^{6}A, N^6-methyladenosine 5′-monosphosphate.

methylation may influence the efficiency or accuracy of polyadenylation. Finally, the availability of a source of m^6A methyltransferase and an assay for methylation will make it possible to purify the enzyme for further functional studies.

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A Continuous Cell-Free Translation System Capable of Producing Polypeptides in High Yield

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A cell-free translation system has been constructed that uses a continuous flow of the feeding buffer [including amino acids, adenosine triphosphate (ATP), and guanosine triphosphate (GTP)] through the reaction mixture and a continuous removal of a polypeptide product. Both prokaryotic (*Escherichia coli*) and eukaryotic (wheat embryos, *Triticum sp.*) versions of the system have been tested. In both cases the system has proven active for long times, synthesizing polypeptides at a high constant rate for tens of hours. With the use of MS2 phage RNA or brome mosaic virus RNA 4 as templates, 100 copies of viral coat proteins per RNA were synthesized for 20 hours in the prokaryotic or eukaryotic system, respectively. With synthetic calcitonin messenger RNA, 150 to 300 copies of calcitonin polypeptide were produced per messenger RNA in both types of continuous translation systems for 40 hours.

EXPRESSION OF ALIEN GENES IN LIVING cells is often subject to a number of limitations. The product polypeptide can be unstable in a given cell, and in some cases the product is toxic to the cell. These and other limitations could be avoided if translation were possible in cell-free systems. Unfortunately, however, different versions of cell-free translation systems that have been previously described have a general shortcoming, namely, a low yield of the polypeptide product. Typically only two to three polypeptide chains are produced per mRNA chain used in such systems (*1, 2*).

We report that a prolonged and effective functioning of a cell-free translation system can be achieved if the reaction products, including synthesized polypeptides, are continuously removed from the reaction mixture, and if the initial concentrations of consumable low molecular weight substrates (ATP, GTP, and amino acids) are continuously restored. This approach has been realized for cell-free translation systems both of prokaryotic (*Escherichia coli*) and eukaryotic

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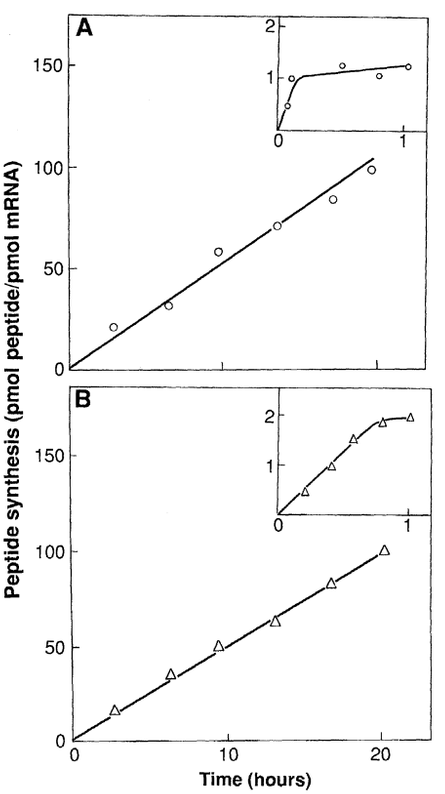

Fig. 1. Kinetics of virus coat protein synthesis in the virus-RNA–directed continuous cell-free translation systems (20 hours). Inset (same axes units): kinetics of protein syntheses in standard cell-free systems of the same composition and volume. (**A**) Synthesis of MS2 coat protein in the *E. coli* system. A 1-ml reaction mixture contained 0.6 nmol of 70*S* ribosomes, 1 mg of S100 protein, 0.6 mg of total tRNA, 0.06 nmol of MS2 phage RNA, 7 μg of pyruvate kinase with a specific activity of 500 U/mg, 50 activity units of ribonuclease inhibitor from human placenta, and 0.1 μg each of aprotinin, leupeptin, and chymostatin in the feeding buffer A, which contained 20 m*M* tris-HCl, *p*H 7.4, 100 m*M* NH_4Cl, 10 m*M* $MgCl_2$, 1 m*M* ATP, 0.2 m*M* GTP, 5 m*M* phosphoenol pyruvate, 25 μ*M* [^{3}H]leucine (specific activity, 52 Ci/mol), and 25 μ*M* each of the other 19 amino acids. The incubation temperature was 37°C. In the continuous system, the feeding buffer A was passed through the Amicon diafiltration cell at a constant rate of 1 ml/hour; the ultrafiltration membrane PM-30 was used for continuously removing MS2 coat protein from the cell. (**B**) Synthesis of BMV coat protein in the wheat system. A 1-ml reaction mixture contained 17 A_{260} absorbance units of S30 extract from wheat (*Triticum* sp.) embryos (Sigma), 0.1 nmol of BMV RNA 4, 64 μg of creatinine phosphokinase with a specific activity of 350 U/mg, 50 activity units of ribonuclease inhibitor from human placenta (Amersham), and 0.1 μg each of aprotinin, pepstatin, and leupeptin in the feeding buffer B, which contained 40 m*M* Hepes, *p*H 7.6, 112 m*M* potassium acetate, 1.9 m*M* magnesium acetate, 0.25 m*M* spermidine, 6 m*M* dithiothreitol, 1.5% glycerol, 2 m*M* ATP, 50 μ*M* GTP, 8 m*M* creatine phosphate, 25 μ*M* [^{3}H]leucine (specific activity, 50 Ci/mol), and 25 μ*M* each of the other 19 amino acids. The incubation temperature was 27°C. In the continuous system, the feeding buffer B was passed through the Amicon cell at a constant rate of 1 ml/hour; the ultrafiltration membrane XM-50 was used for continuously removing BMV coat protein from the cell.